MINIREVIEW / MINISYNTHÈSE

Structure and function of the NHE1 isoform of the Na^+/H^+ exchanger

Emily Slepkov and Larry Fliegel

Abstract: The Na^+/H^+ exchanger is a ubiquitous, integral membrane protein involved in pH regulation. It removes intracellular acid, exchanging a proton for an extracellular sodium ion. There are seven known isoforms of this protein that are the products of distinct genes. The first isoform discovered (NHE1) is ubiquitously distributed throughout the plasma membrane of virtually all tissues. It plays many different physiological roles in mammals, including important functions in regulation of intracellular pH, in heart disease, and in cytoskeletal organization. The first 500 amino acids of the protein are believed to consist of 12 transmembrane helices, a membrane-associated segment, and two reentrant loops. A C-terminal regulatory domain of approximately 315 amino acids regulates the protein and mediates cytoskeletal interactions. Studies are underway to determine the amino acid residues important in NHE1 function. At present, it is clear that transmembrane segment IV is important in NHE1 function and that transmembrane segments VII and IX are also involved in transport. Further experiments are required to elucidate the mechanism of transport and regulation of this multifunctional protein.

Key words: cation transport, intracellular pH, membrane proteins, Na^+/H^+ exchanger.

Résumé : L'antiporteur Na^+/H^+ est une protéine membranaire intégrale qui sert à la régulation du pH et se trouve dans toutes les cellules. Il élimine l'acidité intracellulaire en faisant sortir un proton et entrer un ion sodium extracellulaire. Les sept isoformes connues de cette protéine sont les produits de gènes différents. La première isoforme découverte (NHE1) se trouve dans la membrane plasmique des cellules de tous les tissus. Elle a plusieurs rôles physiologiques chez les mammifères, entre autres, elle joue un rôle important dans la régulation du pH intracellulaire, l'organisation du cytosquelette et les cardiopathies. Les premiers 500 acides aminés de la protéine formeraient 12 hélices transmembranaires, 1 segment associé à la membrane et 2 boucles réentrantes. Un domaine régulateur C-terminal constitué d'environ 315 acides aminés règle la protéine et intervient dans les interactions avec le cytosquelette. Des études sont en cours pour déterminer quels sont les résidus d'acides aminés importants pour la fonction de NHE1. Présentement, il est évident que le segment transmembranaire IV est important pour la fonction de NHE1 et que les segments transmembranaires VII et IX interviennent également dans le transport. D'autres expériences sont nécessaires pour élucider le mécanisme de transport et de régulation de cette protéine multifonctionnelle.

Mots clés : transport de cations, pH intracellulaire, protéines membranaires, antiporteur Na^+/H^+ exchanger.

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Introduction

The mammalian Na^+/H^+ exchangers are a family of integral membrane proteins that mediate the exchange of Na^+ for H^+. These exchangers use the energy of sodium gradients to catalyze the electroneutral exchange of one Na^+ for one H^+. In so doing, most isoforms move protons out of cells, across the plasma membrane. Members of the family of Na^+/H^+ exchangers have a general structure, which consists of a membrane domain of approximately 500 amino acids and a large "tail" of about 300 amino acids (Fliegel 2001; Orlowski and Grinstein 1997). In this review, we describe the family of mammalian Na^+/H^+ exchangers and their physiological roles, focusing on the first known isoform of the protein that has been the most well studied. We also summarize recent findings regarding the mechanism of Na^+/H^+ exchange and the amino acids that are important in the structure and function of the protein.

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Na^+/H^+ exchanger family

To date, seven isoforms of the Na^+/H^+ exchanger (NHE1–NHE7) have been identified (Baird et al. 1999; Sardet et al. 1989; Numata and Orlowski 2001; Numata et al. 1998; Tse

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et al. 1993; Wang et al. 1993). Each isoform represents a distinct gene product with its own specific pattern of tissue expression, membrane localization, kinetic properties, physiological roles, and sensitivities to pharmacological inhibitors (Chambrey et al. 1997; Orlowski 1993; Szabo et al. 2000; Yu et al. 1993). The isoforms share ~20–70% amino acid identity, with calculated molecular weights ranging from ~74 000 to 93 000 (Orolowski and Grinstein 1997). Hydropathy analysis of the various amino acid sequences indicates that the exchangers have similar predicted membrane topologies, with an N-terminal membrane domain consisting of 12 transmembrane helices and a large C-terminal cytoplasmic domain (Wakabayashi et al. 2000*a*).

The NHE1 isoform was discovered first (Sardet et al. 1989) and is the most extensively studied member of the Na^+/H^+ exchanger family. This "housekeeping" isoform is nearly ubiquitous in the plasma membrane of virtually all tissues and is the primary NHE subtype found in the mammalian cardiac cell (Fliegel 2001; Fliegel et al. 1991; Fliegel and Wang 1997). The isoforms NHE2–NHE5 are also localized to the plasma membrane, but they have more restricted tissue distributions. NHE2 and NHE3 are predominantly located in the apical membrane of epithelia. NHE2 is found in larger amounts in the stomach and intestine (Collins et al. 1993; Wang et al. 1993). NHE3 is expressed at high levels in colon and small intestine, with significant levels also present in kidney and stomach (Orlowski et al. 1992). A significant fraction of NHE3 is also present within cells in recycling endosomes (D'Souza et al. 1998). NHE4 is most abundant in stomach epithelium, kidney inner medullar, and hippocampus (Bookstein et al. 1997). NHE5 is expressed predominantly in brain but is also present in other nonepithelial tissues including spleen, testis, and skeletal muscle (Orlowski and Grinstein 1997; Szabo et al. 2000). NHE6 and NHE7 are both localized intracellularly. NHE6 is expressed in metabolically active tissues such as heart, brain, and skeletal muscle, and it was initially reported to be localized to the mitochondria (Putney et al. 2002; Numata et al. 1998). Later reports, however, have suggested that it is localized to an endosomal compartment and is not present in mitochondria (Brett et al. 2002; Nass and Rao 1998). The isoform NHE7 is ubiquitously expressed and is localized predominantly to the *trans*-Golgi network (Numata and Orlowski 2001).

The many physiological functions of the Na^+/H^+ exchanger

Na^+/H^+ exchange activity is centrally important in a variety of physiological processes. The most important role of the mammalian Na^+/H^+ exchanger is to regulate cytosolic pH. The exchanger protects cells from intracellular acidification: mutant cell lines devoid of Na^+/H^+ exchange activity are extremely sensitive to acidosis (Grinstein et al. 1989; Pouyssegur et al. 1984). The Na^+/H^+ exchanger is activated by decreased intracellular pH, so when acidosis occurs the increased activity of the protein compensates, raising intracellular pH to "normal" values. The Na^+/H^+ exchanger also regulates sodium flux and cell volume after osmotic shrinkage (Grinstein et al. 1989; Shrode et al. 1996). When cells are exposed to hyperosmotic solutions, the Na^+/H^+ exchanger responds with rapid increases in activity that result in cytosolic alkalinization. This response comprises part of a regulatory increase in cell volume, which compensates for the shrinkage caused by the external hyperosmolarity (Bianchini et al. 1995).

In addition to its role in regulating cellular pH and volume, the Na^+/H^+ exchanger also initiates shifts in intracellular pH that stimulate changes in the growth of cells or alter their functional state (Grinstein et al. 1989; Hoffmann and Simonsen 1989). For example, it has long been known that the Na^+/H^+ exchanger is important in the growth of tumors because tumor cells deficient in Na^+/H^+ exchange activity either fail to grow or show severely retarded growth when implanted into immune-deficient mice (Rotin et al. 1989). More recently, it has been demonstrated that cellular alkalinization resulting from NHE1 activation is a key step in oncogenic transformation and is necessary for both the development and maintenance of a transformed phenotype (Reshkin et al. 2000). It is also known that mitotic stimulation of cells is associated with increased expression of the Na^+/H^+ exchanger protein. This increased expression may aid in the elevation and maintenance of intracellular pH during cell growth (Besson et al. 1998).

Increased activity and increased levels of expression of the Na^+/H^+ exchanger are also important in cell differentiation. A number of different treatments result in increased mRNA levels for NHE1, including treatments that cause cellular differentiation (Rao et al. 1991, 1992). During the differentiation of human leukemic cells (HL-60), transcription of the NHE1 gene increases 18-fold and NHE1 protein levels increase sevenfold (Rao et al. 1991, 1992). More recently, we have shown that there is a transient increase in transcription of the NHE1 gene both during differentiation of P19 cells induced by retinoic acid and when L6 cells differentiate from myoblast to myotubes (Yang et al. 1996). These observations suggest that the Na^+/H^+ exchanger plays an important role in cellular differentiation. It has been suggested that increased exchanger activity during differentiation is important in enabling the differentiation to occur, at least in some cell types (such as P19 cells) (Alvarez et al. 1989; Hazav et al. 1989; J. Wang et al. 1997). However, since this requirement does not appear to be universal, the importance of the Na^+/H^+ exchanger may be diminished where other proteins are important in pH regulation (Vairo and Hamilton 1993).

pH regulation activity by the Na^+/H^+ exchanger plays a significant part in mediating the damage that occurs to the human myocardium during ischaemia and reperfusion. During cardiac ischaemia, protons accumulate and the intracellular pH drops. During the ischaemia and the subsequent reperfusion, the Na^+/H^+ exchanger removes intracellular protons and this results in an accumulation of intracellular Na^+. The accumulation of Na^+ either reduces extrusion of Ca^{2+} by the Na^+/Ca^{2+} exchanger or even reverses this bidirectional exchanger (Karmazyn 1996*a*, 1996*b*, 2001; Karmazyn et al. 1999). Excess Ca^{2+} therefore accumulates inside the cell and causes cell necrosis, contracture, and cardiac arrhythmias. Amiloride derivatives, which inhibit the Na^+/H^+ exchanger, are successful in blocking this cycle of damage to the myocardium. In addition, a new class of anti-ischaemic, guanidinium-derivative compounds, including HOE694,

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HOE642, and cariporide, has also proven useful for this purpose (Karmazyn 1996*a*, 1996*b*; Avkiran and Yasutake 1996).

Recently, the Na^+/H^+ exchanger has also been shown to play an important role in myocardial hypertrophy. Hypertrophy of the myocardium is an early, maladaptive response to congestive heart failure and its attenuation is a primary objective for therapeutic treatments. Recent studies have shown that inhibition of Na^+/H^+ exchanger activity can prevent myocardial hypertrophy (Yoshida and Karmazyn 2000; Kusumoto et al. 2001). The mechanism by which this occurs has not yet been determined, but it likely involves either the proton or sodium fluxes resulting from Na^+/H^+ exchanger activity.

In addition to regulating cytosolic pH, NHE1 also acts as a structural anchor that is involved in organization of the cytoskeleton (Denker and Barber 2002). This structural role of NHE1 is independent of its function as an ion exchanger. NHE1 acts as an anchor for actin filaments that control the integrity of the cortical cytoplasm. The anchoring occurs via structural links between NHE1 and the actin-binding proteins ezrin, radixin, and moesin (Denker et al. 2000). The importance of NHE1 as a structural anchor is shown in fibroblasts expressing NHE1 mutants that have disrupted ERM binding. These cells have impaired organization of focal adhesions and actin stress fibers, and they have an irregular cell shape (Denker et al. 2000). Overall, structurally, NHE1 is thought to determine membrane integrity and cell shape and to restrict transmembrane proteins to localized microdomains (Putney et al. 2002). Furthermore, the structural role of NHE1 may enable actin-dependent regulation of NHE1 activity and could result in a restricted distribution of NHE1 enabling localized H^+ efflux (Putney et al. 2002).

Evidence suggests that the Na^+/H^+ exchanger might also play a physiological role in moderating apoptosis, although this varies with cell type. For example, pH regulation by the Na^+/H^+ exchanger elicits a novel response in cytokine-dependent, pro-β-cell lines. Specifically, following withdrawal of cytokines, the Na^+/H^+ exchanger mediates an elevation of intracellular pH (Khaled et al. 2001). This triggers an apoptotic pathway, allowing the proapoptotic protein Bax to translocate to the mitochondria (Khaled et al. 1999). Studies in human leukemic cells also suggest that the Na^+/H^+ exchanger plays a role in apoptosis. However, in these cells, inhibition of the Na^+/H^+ exchanger elicited a decreased intracellular pH, and this was followed by the rapid induction of apoptosis (Rich et al. 2000). These results suggest that, under appropriate conditions, inhibition of the Na^+/H^+ exchanger may be a potential route for antileukemic therapy. The roles that the Na^+/H^+ exchanger plays in apoptosis remain to be characterized in various cell lines and tissues.

Na^+/H^+ exchanger pharmacology

All isoforms of the Na^+/H^+ exchanger are inhibited by the diuretic compound amiloride and its analogues and by novel benzoyl guanidinium compounds such as HOE694 and cariporide (Counillon et al. 1993*b*). Comparisons of the different isoforms of the Na^+/H^+ exchanger show that they have differing affinities for these inhibitors, with the following order of sensitivity under similar experimental conditions: NHE1 ≥ NHE2 > NHE5 > NHE3 (Orlowski and Grinstein 1997). NHE4 and NHE6 are both insensitive to amiloride (Putney et al. 2002). Since NHE1 is the primary isoform of the Na^+/H^+ exchanger in mammalian cardiac cells (Fliegel and Dibrov 1996; Fliegel et al. 1991, 1993*a*; Orlowski et al. 1992), the selective properties of these inhibitors for this isoform have been exploited therapeutically for treatment of cardiac ischaemia and reperfusion injuries. A recent clinical trial, the "GUARDIAN" trial, evaluated the ability of the inhibitor cariporide to afford protection to a variety of subjects at risk for ischaemic heart disease. The drug showed beneficial effects in only one subgroup of patients, those with coronary artery bypass graft surgery (Avkiran and Marber 2002). The results of this and other trials suggest that the cardioprotective effects of inhibiting the Na^+/H^+ exchanger are apparent only if the inhibition occurs before the onset of myocardial ischaemia (Avkiran and Marber 2002). Overall, inhibition of the Na^+/H^+ exchanger appears to offer a reasonable strategy for treatment of at least some forms of myocardial heart disease.

Although the Na^+/H^+ exchangers have been extensively studied, little is known about how these antiporters actually interact with their inhibitors and how the inhibitors function in relation to the binding and transport of Na^+ and H^+. For some time, it was thought that Na^+ and the inhibitors of the Na^+/H^+ exchanger bind at a common site because amiloride and HOE694 both competitively inhibit Na^+ binding (Harris and Fliegel 1999). However, there is also evidence to the contrary, as several studies have demonstrated that the Na^+-binding site and the inhibitor-binding site can be altered independently of one another (Orlowski and Kandasamy 1996; Wang et al. 1995; Yun et al. 1993). In addition, under chloride-free buffer conditions, amiloride and its derivatives inhibit transport noncompetitively (Ives et al. 1983; Warnock et al. 1988). Considering all of these findings, it now appears that Na^+ and inhibitors of the Na^+/H^+ exchanger compete for physically distinct binding sites that can sometimes function cooperatively with respect to ion exchange. It may be that the inhibitor-binding site partially overlaps the Na^+-binding site or that binding of the inhibitor alters the Na^+-binding site (Harris and Fliegel 1999). Further research into the exact location of the inhibitor- and Na^+-binding sites could lead to the development of more potent and selective inhibitors of the protein.

General structure of the NHE1 isoform of the Na^+/H^+ exchanger

Despite more than 40 years of investigation into the function of the Na^+/H^+ exchanger, much remains unknown about this critically important protein. It is known that the membrane domain of the exchanger is both necessary and sufficient for ion transport, while the cytosolic domain is involved in the regulation of ion-exchange activity (Wakabayashi et al. 1992). Recently, using substituted cysteine accessibility analysis, Wakabayashi et al. (2000*a*) determined that NHE1 has a novel membrane topology. As illustrated in Fig. 1, in addition to confirming that NHE1 has 12 transmembrane helices, with both the N- and C-termini located in the cytosol, these authors also identified a membrane-associated segment and two

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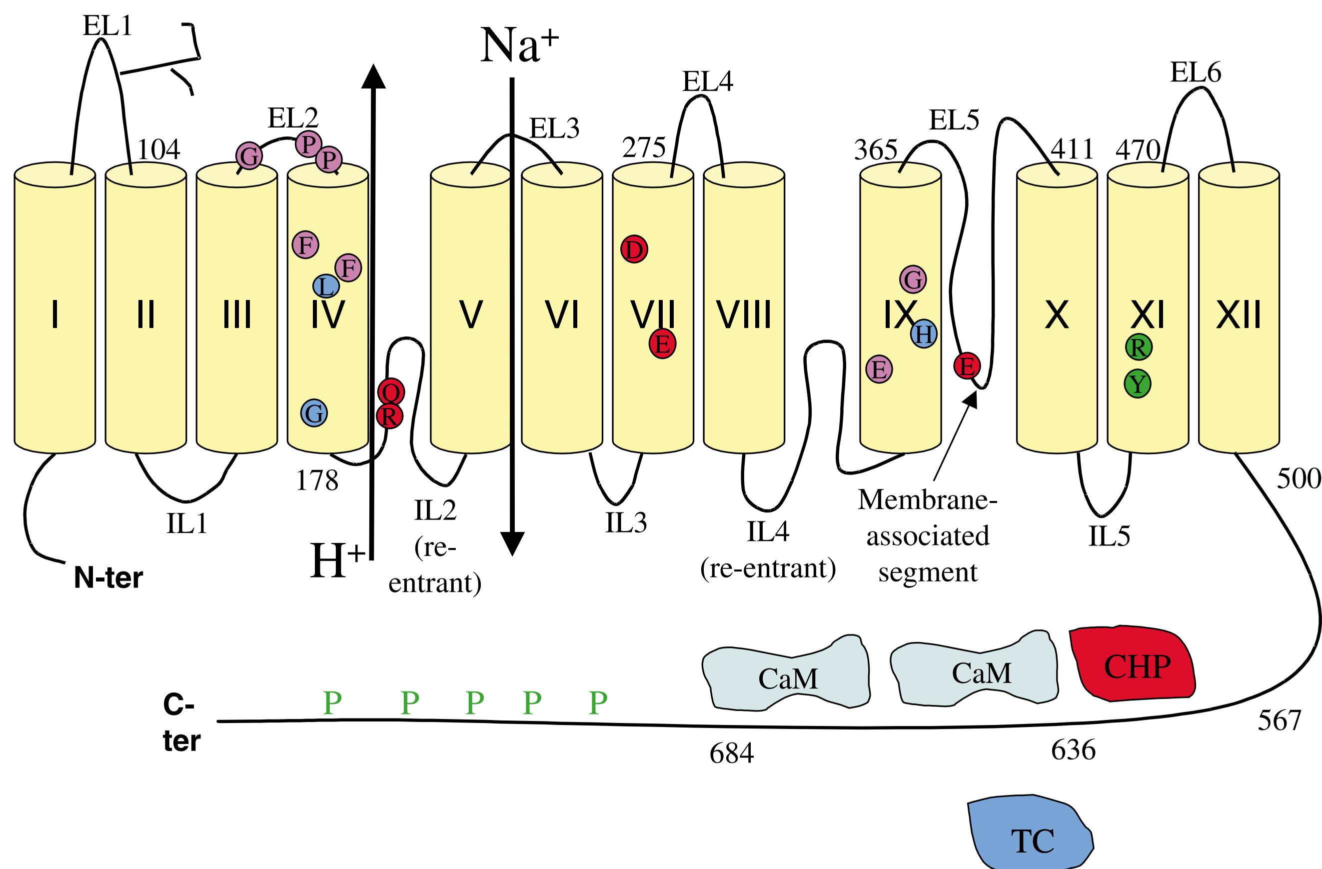
Fig. 1. Model of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. The topology of the protein is illustrated after Wakabayashi et al. (2000*a*). Amino acid residues known to be important in structure and function are illustrated. Regions of the cytoplasmic domain important in regulation or protein–protein interaction are illustrated. Purple circles, residues implicated in both ion binding and transport and inhibitor binding; red circles, residues implicated in ion binding and transport; blue circles, residues implicated in inhibitor binding; green circles, residues implicated in Na^+/H^+ exchanger folding and targeting to the plasma membrane. IL, intracellular loop; EL, extracellular loop. The positions of reentrant intracellular loops and the membrane-associated segment are illustrated. The associated proteins calmodulin (CaM), calcineurin homologous protein (CHP), and tescalcin (TC) are illustrated in their approximate known binding sites. The binding site of TC is not known. "P"s indicate the approximate sites of phosphorylation of the cytosolic tail of the protein. Numbering indicates the putative positions of the amino acids within the model.



reentrant loops within the protein. In terms of quaternary structure, it has been demonstrated that NHE1 forms homodimers (Fafournoux et al. 1994; Fliegel et al. 1993*b*). However, the NHE1 monomer is the minimal functional unit required for Na^+/H^+ exchange activity and it remains unclear whether the dimerization is necessary for activity (Fafournoux et al. 1994).

Regulation of the activity of NHE1

The activity of the NHE1 isoform of the Na^+/H^+ exchanger is closely regulated. The major regulatory stimulus for this protein is intracellular acidosis, which is negligible under normal physiological conditions. However, if and when intracellular pH does decrease, the exchanger is rapidly activated (Karmazyn et al. 2001). In addition to responding to intracellular protons, NHE1 can be activated by extrinsic factors such as hormones and growth factors. These factors activate NHE1 by shifting its pH dependence to a more alkaline range, causing it to have a greater than "normal" activity at alkaline pH (Fliegel 2001). Much of the shift in pH dependence is accomplished mainly through phosphorylation of the exchanger's C-terminal regulatory domain, near the distal end of the tail (amino acids 656–815 in human NHE1) (Moor and Fliegel 1999). The regulation of the Na^+/H^+ exchanger by phosphorylation is complex and appears to vary with cell type. Several kinases are thought to regulate NHE1 activity. To date, it is known that phosphorylation by mitogen-activated protein kinase (Moor and Fliegel 1999; Moor et al. 2001; H. Wang et al. 1997), Rho kinase (Tominaga et al. 1998), Nck-interacting kinase (Yan et al. 2001), and $p90^{rsk}$ (Moor and Fliegel 1999; Takahashi et al. 1997) results in activation of the exchanger. In contrast, protein kinase p38 is reported to inhibit the exchanger in some cell types (Kusuhara et al. 1998) and stimulate it in others (Khaled et al. 2001). Other kinases, including protein kinase C and protein kinase D, regulate the exchanger but do not appear to phosphorylate it directly (Fliegel et al. 1992; Haworth et al. 1999; Snabaitis et al. 2000; H. Wang et al. 1997). It is not yet known how phosphorylation of the Na^+/H^+ exchanger mediates the increased exchange activity.

The Na^+/H^+ exchanger is also regulated via interactions with a number of regulatory proteins (Fig. 1). The cytoplasmic, C-terminal tail of NHE1 has two domains that are capa-

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ble of binding calmodulin with high affinity (CaM-A, K_d ~ 20 nM) and low affinity (CaM-B, K_d ~ 350 nM), respectively (Bertrand et al. 1994). The high-affinity site is believed to be important in regulating NHE1 activity, with the current model suggesting that at basal intracellular Ca^{2+} levels the unoccupied CaM-A site exerts an autoinhibitory effect that is relieved upon Ca^{2+}–calmodulin binding (Fliegel 2001; Wakabayashi et al. 1994). A second Ca^{2+}-binding protein that interacts with NHE1 is calcineurin B homologous protein (CHP) (Lin and Barber 1996). Overexpression of CHP prevents stimulation of NHE1 activity. Thus, it appears that CHP could act as a negative regulatory protein, which binds to NHE1 constitutively in the resting state, maintaining a reduced transport activity (Putney et al. 2002). Recently, a novel regulatory protein has been found to bind to the Na^+/H^+ exchanger. This protein, called tescalcin, is a calcium-binding protein that is implicated in differentiation and that has homology to CHP. It was found to bind to the carboxyl terminal of the Na^+/H^+ exchanger and to colocalize with the Na^+/H^+ exchanger in cellular lamellipodia. Its function is not yet clear, although it may have a role similar to that of CHP (Mailander et al. 2001). Finally, the protein HSP70 can also bind directly to the C-terminal regulatory domain of NHE1 (Silva et al. 1995), but this interaction is likely involved in folding and processing of the antiporter rather than in regulation.

The complex interplay among components that occurs in regulation of the Na^+/H^+ exchanger by both phosphorylation and protein–protein interaction is still not well understood. The effects of phosphorylation on the action of the regulatory proteins, and vice versa, remain to be determined. Importantly, however, results to date suggest that the regulation of NHE1 varies from one cell type to another.

Regulation of the expression of the Na^+/H^+ exchanger

The factors involved in regulating expression of the Na^+/H^+ exchanger are only now starting to be understood. In 1991, Miller et al. (1991) isolated the first genomic clone of the human Na^+/H^+ exchanger gene. Since then, the promoter of the mouse NHE1 gene has been cloned in our laboratory (Dyck et al. 1995) and the promoter of the rabbit NHE1 gene has also been cloned (Blaurock et al. 1995). Elements of the NHE1 promoter that are important in regulating gene expression have been analyzed in several cell types, and it appears that the regulation of expression varies from one cell type to another. The transcription factor AP-1 is involved in regulation of NHE1 expression in cultured cells from the renal proximal tubule (Horie et al. 1992). Four proximal regions of the human promoter were reported to be important in NHE1 expression in hepatic and smooth muscle cells (A–D, from most proximal to distal relative to the start site), and deletion or substitution of nucleotides within the D region caused decreases in promoter activity (Kolyada et al. 1994). This region of DNA was shown to bind rat liver nuclear factor(s), possibly C/EBP (Kolyada et al. 1994, 1995).

The mouse NHE1 promoter has been analyzed in more detail than either the human or rabbit promoter. Deletion of regions of the promoter upstream of an AP-2-like site reduced basal activity of the promoter by 70% in fibroblasts (Dyck et al. 1995) and by 40% in P19 embryonal carcinoma cells (Dyck and Fliegel 1995). Gel mobility shift analysis and transfection with an AP-2 expression plasmid showed that the transcription factor AP-2, or an AP-2-like protein, binds to this region of the NHE1 promoter and is important in expression. More recently, a novel poly (dA:dT) region of the promoter was shown to be involved in regulation of NHE1 expression in L6 and NIH 3T3 cells (Yang et al. 1996). In addition, we have shown that serum and growth factors stimulate promoter activity in cardiomyocytes and fibroblasts. In both of these cell types, more distal elements of the promoter (0.8–1.1 kb) are involved (Besson et al. 1998), and further, COUP transcription factors (COUP-TFs) act at this distal region to regulate expression of the Na^+/H^+ exchanger gene. COUP-TFs are orphan receptors that are important in embryonic development and in neural cell determination. The distal element of the NHE1 promoter was responsive to the expression of COUP in NIH 3T3 (fibroblast) cells, suggesting that COUP-TFs may play a role in regulating expression of the Na^+/H^+ exchanger during development (Fernandez-Rachubinski and Fliegel 2001). In keeping with this suggestion, we have recently shown that expression of the Na^+/H^+ exchanger is regulated during embryonic development. The level of NHE1 gene transcription is highest, in utero, in the heart and liver at 12 days of development. It then declines to a much lower level immediately prior to birth (Reider and Fliegel 2002).

Regulation of the NHE1 promoter affects both mRNA levels and production of NHE1 protein. Obviously, the amount of NHE1 protein in a cell will affect the maximal velocity of ion exchange, and so, not unexpectedly, a number of environmental stimuli have been shown to affect the level of mRNA for NHE1, the amount of the protein itself, and Na^+/H^+ exchange activity in the myocardium. For example, both NHE1 message levels and the activity of NHE1 are greater in the cardiac tissue of newborn animals compared with adult animals (Chen et al. 1995; Haworth et al. 1997; Meno et al. 1989). This increased expression of NHE1 may explain why ischaemia and acidosis have a reduced inhibitory effect on cardiac contractility in newborn rabbit myocardium compared with adult myocardium (Meno et al. 1989). Stimuli such as pressure overload also increase NHE1 expression in the cardiovascular system (Takewaki et al. 1995).

As discussed earlier, Na^+/H^+ exchange activity is a mediator of damage that occurs to the myocardium during ischaemia and reperfusion. Because of this, several groups have examined the effects of ischaemia on the expression of NHE1. In isolated perfused hearts, ischaemia caused levels of mRNA for the Na^+/H^+ exchanger to increase. In addition, subjecting isolated cardiac cells to external acidosis resulted in increased activity of the exchanger (Dyck et al. 1992, 1995; Gan et al. 1999). It has also been noted that NHE1 message and protein levels are both increased after myocardial infarction (Sandmann et al. 2001). Future studies may explore more directly the mechanisms by which these clinical diseases increase NHE1 message and protein levels.

Mechanisms of Na^+/H^+ exchange

Little is known about the overall mechanism behind cation

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transport by NHE1. However, several studies have identified specific amino acids that are important in inhibitor binding and in ion transport. Specifically, residues in transmembrane segments IV, VII, IX, and XI, in the membrane-associated segment, and in the two reentrant loops have been implicated (see Fig. 1). The majority of residues implicated in ion transport and inhibitor binding are located in transmembrane segment IV. In 1993, Counillon et al. (1993*a*) found that a Phe165Tyr mutation in transmembrane segment IV of the hamster NHE1 sequence, which corresponds to Phe161 in the human sequence, causes both a 40-fold increase in resistance to the amiloride derivative N^5-methyl-N^5-propylamiloride (MPA) and a three- to fourfold decrease in Na^+ transport rate. This indicates that Phe165 affects both amiloride binding and the V_{max} for Na^+ transport. In the same study, these authors found that a Leu167Phe mutation in transmembrane segment IV, which corresponds to Leu163 in the human sequence, causes a 30-fold increase in resistance to MPA with no effect on Na^+ transport. In 1997, using random mutagenesis, the same group found that a Gly174Ser mutation in NHE1 causes a modest 3.3-fold increase in resistance to amiloride, with no effect on Na^+ transport (Counillon et al. 1997). They also made an NHE1 double mutant (Leu163Phe/Gly174Ser), which they found to have a strongly reduced affinity for various inhibitors and a twofold decrease in sodium affinity, further implicating transmembrane segment IV as important in ion binding and transport. Recently, a Phe162Ser mutation, also in transmembrane segment IV, was found to cause a dramatic decrease in affinity for cariporide and a 10-fold decrease in affinity for Na^+ (Touret et al. 2001). Finally, two other substitution mutations have been investigated: a single Gly152Ala mutation, which corresponds to Gly148 in the human sequence, and a double Pro157Ser/Pro158Phe mutation, which corresponds to Pro153/Pro154 in the human sequence. In both cases the mutated residues are located in the second exomembrane loop at the N-terminal end of transmembrane segment IV, and they both modestly reduce the drug sensitivity and catalytic turnover of the exchanger (Khadilkar et al. 2001). Overall, these studies provide a strong case for the involvement of transmembrane segment IV in the ion binding and transport properties of NHE1.

In contrast with transmembrane segment IV, less is known about other regions of NHE1 that may be involved in its function. We have shown that residues Glu262 and Asp267 are essential for NHE1 activity, and so transmembrane segment VII is clearly also involved in the ion binding and transport capabilities of NHE1 (Murtazina et al. 2001). The effects of these mutations do not arise from alterations in the structure of NHE1, since mutating nearby residues has no effect, mutant proteins are properly targeted to the plasma membrane, and limited proteolytic digestion shows no differences between the wild-type and mutant exchangers (Murtazina et al. 2001). In addition, substitution of mutated Glu262 and Asp267 with alternative acidic residues restores Na^+/H^+ exchanger activity, further confirming that the introduced mutations were not affecting the overall conformation of NHE1 (Murtazina et al. 2001).

Transmembrane segment IX also appears to be involved in NHE1 function. Specifically, it seems to play a role in amiloride binding, since mutating His349 to glycine or leucine increases resistance to amiloride, and mutating His349 to tyrosine or phenylalanine decreases resistance to amiloride (Wang et al. 1995). Moreover, in 1996, Orlowski and Kandasamy (1996) found that interchanging a 66 amino acid segment of NHE1, which contains transmembrane segment IX and its adjacent loops, with the same segment in NHE3 caused reciprocal alterations in the sensitivities of these isoforms to amiloride, ethylisopropylamiloride, HOE694, and cimetidine (Orlowski and Kandasamy 1996). Again, the chimeric NHE1 mutants retained their normal Na^+ transport properties. More recently, two residues within transmembrane segment IX of rat NHE1, Glu350 and Gly356, which correspond to Glu346 and Gly352 in human NHE1, respectively, were identified as major determinants of drug sensitivity (Khadilkar et al. 2001). Mutation of these residues does not appreciably affect Na^+ affinity, but it markedly decreases the catalytic turnover of the transporter.

Finally, two recent papers have suggested that transmembrane segment XI plays a role in NHE1 function. The first of these describes a novel topological model of NHE1, which is based on substituted cysteine accessibility analysis. In this study, a number of cysteine mutants in transmembrane segment XI showed altered function, suggesting that these residues may be involved either in ion transport or in proper targeting to the plasma membrane (Wakabayashi et al. 2000*a*). In the second paper, localization studies determined that two of these mutants, Tyr454Cys and Arg458Cys, are retained in the endoplasmic reticulum (Wakabayashi et al. 2000*b*). Although these results seem to indicate some importance for transmembrane segment XI, it remains unclear at this time whether this transmembrane segment is directly involved in NHE1 function or if it is only required for proper folding and targeting of the transporter.

A number of the residues that are implicated in NHE1 function are not located in transmembrane helices. Specifically, these residues are located in intracellular loops 2 and 4 (IL2 and IL4) and in the membrane-associated segment (Fig. 1). The major evidence implicating IL2 and IL4 comes from studying the accessibility of cysteine in mutated amino acids in these loops. It was found that parts of IL2 and IL4 are accessible from both sides of the membrane (Wakabayashi et al. 2000*a*). In addition, treating the mutants Arg180Cys and Gln181Cys (both mutations located within IL2) with the membrane-impermeant sulfhydryl reagent 2-trimethylammoniumethyl-methanethiosulfonate severely inhibits transport. The authors hypothesized that IL2 and IL4 are located in a pore-lining region of NHE1 that is involved in ion transport (Wakabayashi et al. 2000*b*). Further evidence for the importance of these loops comes with the prediction that IL2 and IL4 contain the putative H^+-sensor region of NHE1 (Kinsella et al. 1998).

Some functional importance for the membrane-associated segment of NHE1 is thought to be likely because this segment contains several polar amino acids and is reminiscent of the selectivity filter of potassium channels (Murtazina et al. 2001). Recently, our group demonstrated that Glu391, located within the membrane-associated segment, is important for activity (Murtazina et al. 2001). Again, mutation to an alternative acidic residue restored Na^+/H^+ exchanger activity, indicating that the effects of mutating Glu391 almost certainly do not arise from an altered structure of NHE1. Thus,

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the membrane-associated segment also plays a role in the ion binding and transport properties of NHE1.

Summary and significance

The Na^+/H^+ exchanger is a membrane protein with diverse physiological functions. It is important in cell proliferation, in heart disease, in apoptosis, and in cellular differentiation. Although a great deal of research has been done on the Na^+/H^+ exchanger, there are many remaining questions, including unravelling the processes involved in its regulation. It also remains to be determined whether inhibitors of the Na^+/H^+ exchanger will be useful in clinical treatments for heart disease. The mechanism by which the Na^+/H^+ exchanger transports ions and interacts with inhibitors remains unknown. However, several key amino acid residues have been implicated as being important in the function of NHE1, and transmembrane segments IV, VII, and IX appear to be particularly likely to interact with transported cations. In addition, it is apparent that the C-terminal cytoplasmic domain interacts with, and regulates, the transport activity of the N-terminal membrane domain, but the mechanism behind this is also unknown. Additional mutagenesis studies will help to elucidate the mechanisms of NHE1 ion transport. Overall, it is clear that while a great deal of fundamental knowledge about the Na^+/H^+ exchanger has been gained, many questions are as yet unanswered and future research is required to increase our understanding of this important membrane protein.

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